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A REFERENCE METHOD FOR DETECTING *GIARDIA* CYSTS IN WATER

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16. ABSTRACT

Giardia detection methodology for water samples is relatively new, having been in existence for less than 10 years. Current methodology is based upon microscopic examination of concentrates from large volume water samples. This procedure is tedious and time-consuming and it fails to provide important information on the viability of detected cysts. The method for the 16th edition of Standard Methods is described along with the applications and limitations of the methodology.

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A REFERENCE METHOD FOR DETECTING GIARDIA CYSTS IN WATER

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INTRODUCTION

The waterborne transmission of giardiasis has been firmly established with 80 outbreaks having been reported in the United States from 1965 through 1983 (1,2). The outbreaks are continuing and more than 350,000 people in Pennsylvania were on boil-water orders in 1984 because of concern about giardiasis (3). Until 1975, the evidence for waterborne transmission was entirely epidemiological--no one had successfully detected Giardia cysts in water samples. In that year, the Centers for Disease Control (CDC) used a swimming pool filter, containing sand as the filtering medium, to collect 10 samples representing more than one million liters of raw Rome, New York water (4). Portions of the sediment from two of the 10 samples caused infection in beagle puppies and one cyst was detected microscopically. The sampling equipment was bulky and heavy, requiring transport by truck or air freight, and the field collection of the filter backwash into 55-gallon drums was difficult and cumbersome.

The CDC discontinued use of their swimming pool filter method in 1976 when the USEPA Health Effects Research Laboratory (HERL) in Cincinnati developed a portable sampling device and associated methodology (5). With the HERL methodology, cysts were demonstrated for the first time in the raw water and distribution system of a water supply during the Camas, Washington outbreak in May, 1976. The method involved concentrating particulates from water samples on microporous yarn-wound orlon filters, extracting the filters, purification steps to separate the cysts from interfering debris, and microscopic examination of the concentrate.

It should be recognized that a cultural method for the detection, isolation, and enumeration of Giardia organisms from water is not currently available. Cultures of Giardia trophozoites can be established from clinical specimens and maintained in the laboratory (6,7). However, this procedure is far from routine and it may not be practical, even with relatively pure source material from duodenal aspirates, until additional information is available on the nutritional requirements of the organisms (8). Developing a cultural method for Giardia cysts in water samples may be an even more formidable task than it is for trophozoites in clinical specimens because of the wide variety of competing organisms, including bacteria, viruses, and aquatic protozoa, that must be controlled. Currently, detection methodology for Giardia in water is dependent upon microscopic examination or upon animal feeding of concentrates. Because of the difficulty, expense or unavailability of suitable animal models, the methodology has historically focused on microscopic detection methods.

"STANDARD" METHODS

The method developed by HERL, variously known as the EPA method, the HERL method, or the orlon filter method, subsequently appeared in the 15th edition of Standard Methods (9). This method is NOT a standard method. It appeared in Section 912, Detection of Pathogenic Microorganisms. Methods included in this section are intended as a starting point for the investigator with a need to sample for one or more of the included pathogens. For many of these pathogens, the methdology may be rapidly changing and there may be a lag period of two or more years from the writing of the method to publication in Standard Methods. As indicated above, Giardia methodology is new, having been in existence for less than 10 years, and by the time the 15th edition became available, there had been significant changes in the methodology.

The original impetus for developing a method was to assist in outbreak investigations. The qualitative methodology that was developed did prove useful in some such investigations. However, the need now has shifted to a quantitative monitoring method that may be used to satisfy a possible maximum contaminant level (MCL) or treatment regulation. Additionally, a method of this type might be useful in determining whether or not a variance or exemption would be appropriate for a given water supply.

A REFERENCE METHOD

Recognizing the shifting priorities and the need for some form of standardization, HERL convened a small workshop on Giardia methods in the fall of 1980 (10). Participants in the workshop consisted of investigators who had been working to some extent with methods for detecting Giardia cysts in water samples. The product of the workshop was a consensus method and it was suggested that laboratories with a need or desire and the ability to perform the test use this method as a starting point or reference. This reference method will appear in the 16th edition of Standard Methods. The details of the method are presented below and a flow chart is given in Fig. 1.

a. Sampling:

Collect samples of the raw water source, at the treatment plant before disinfection, and from the distribution system. The total number of samples will depend on the study objectives and available resources. Collect a sample using an apparatus consisting of an inlet hose, plastic filter holder with 23-cm long yarn-wound filter (1-μm porosity), outlet hose, water meter, and a limiting orifice flow control device with a flow rate of 6.3×10^{-5} m^3/sec (1 GPM). The yarn may be orlon, polypropylene, or other suitable material that does not release fibers during subsequent processing. Components of the apparatus need not be sterile, but thoroughly drain and rinse the equipment between samples. Use aseptic technique during sample collection to protect the sample collector and to prevent sample cross-contamination. A line pressure of 100 to 130 kPa (15-18 psi) is satisfactory; if water under pressure is unavailable, use a pump. The volume of water to be sampled depends on the intent of the investigation; a minimum sample size of 380 L is suggested.

To collect a sample, connect inlet hose to an appropriate sampling tap. The direction of water flow is from the outside to the inside

of the yarn-wound filter cartridge. Record time and meter reading and open sampling tap. Collect sample, turn off sampling tap, record time and meter reading, and disconnect sampling apparatus. Take care to maintain opening on the inlet hose above the level of opening on outlet hose to prevent filter backwashing loss of particulate matter. Drain residual water as completely as possible from the sampling apparatus. When unit is completely drained, open filter holder, aseptically remove filter cartridge, place it in a labelled plastic bag, and seal bag. If additional samples are to be collected with the same filter device, thoroughly rinse the influent hose and filter holder with the water to be sampled before installing another filter cartridge. Refrigerate samples or place on wet ice as soon as possible after collection. Transport to the laboratory on wet ice and process as soon as practical but within 48 hours. Do not freeze. Minimize shipping and storage times.

b. Sample Processing:

1. Extraction. Handle filter samples aseptically. Using a razor knife, separate the filter fibers from the support core by making a longitudinal cut down the entire length of the filter cartridge. Cut the fibers into two or more approximately equal portions consisting of an outer and an inner layer. There often will be a fairly distinct line of demarcation based on the depth to which sample particulate matter has penetrated the filter cartridge. Alternatively, locate the end of yarn on the outside of the cartridge, unwind the fiber, and divide into equal portions. Extract each portion separately with 1 L distilled water by kneading or by shaking for approximately 10 minutes. Express fluid from the fiber portions and combine all fluid. If the fiber mat retains significant amounts of particulate matter, repeat the extraction process on each portion with fresh liter volumes of water until the fibers appear clean. If further processing is not possible at this point, preserve the extract by adding a sufficient volume of 37% (V/V) formaldehyde to produce a final concentration of 2% (V/V) formaldehyde. Refrigerate the preserved extract until concentration.

2. Extract Concentration. Concentrate the filter extract by letting the extract settle in the refrigerator overnight or by centrifuging at 600 x g for 5 minutes. If available, use an algal centrifuge. Decant or aspirate the supernatant fluids and resuspend the pellets in a volume of 2% (V/V) formaldehyde equal to the total volume of the combined sediments. If little or no sediment is present, suspend the sediments in 10 ml of 2% (V/V) formaldehyde. [The algal centrifuge produces a total volume of 7 ml of supernatant fluid and sediment. Add 3 ml 2% (V/V) formaldehyde and wash by centrifugation.] Recentrifuge the combined sediments for 5 minutes at 600 x g. If the sediment volume is equal to or less than 1 ml, continue processing by decanting or aspirating the entire supernatant. Discard supernatant. If the sediment volume is >1 ml, resuspend it in the supernatant fluid, transfer a volume of suspension equivalent to 1 ml of sediment to a 15 ml tube and again centrifuge for 5 minutes at 600 x g. Decant and discard the supernatant.

Add 2 to 3 drops of Lugol's iodine [dilute stock solution 1:5 with sp. g. 1.20 zinc sulfate ($ZnSO_4$) solution] to the pelleted material and mix with an applicator stick. Add 5 ml $ZnSO_4$ solution (sp. g. 1.20) and mix on a vortex mixer. Bring volume up to the top of the tube with additional $ZnSO_4$ swinging bucket rotor, centrifuge for 3 minutes

at 650 x g. Touch a clean, grease-free, #1 22 x 22 mm coverslip to the meniscus and let tube and contents remain static for 2 to 3 minutes after placement. Be careful to handle the coverslip by the edges. Carefully lift coverslip straight up, place on a slide, and seal coverslip with vaspar.

3. Microscopic Examination. Scan entire coverslip area with a 10X objective on a bright-field microscope using reduced light. Identify suspect organisms using a 45X objective and an ocular micrometer. Identification must be made by an individual with a demonstrated proficiency for recognizing and differentiating intestinal protozoa. Take care not to confuse Giardia with yeast, diatoms, Coccidia, and other organisms. Positive identification of Giardia requires observing structures of the right size and shape and having at least two internal morphological characteristics. Examine sediment from the entire sample in the above manner before declaring a sample to be negative.

c. Reporting Results:

Report dimensions of Giardia cyst-like structures larger than 8 µm and less than 19 µm. In addition, report any internal morphological features observed. Photomicrographs of suspect cysts may be useful in documenting or corroborating identification. Record and report non-Giardia microorganisms detected.

d. Cultivation:

No in vitro method for cultivating Giardia from the cyst stage is currently available. Trophozoites may be cultured; however, it is less likely that trophozoites would be present in water supplies in detectable numbers or that they would survive long.

DISCUSSION

The above method, that will appear in the 16th edition of Standard Methods, again is NOT a standard method. It is a tentative method and should be used as a reference or starting point. This method, or modifications of it, have been used by a small number of laboratories in the last four years. The method is workable in the hands of trained individuals but it is not without problems: it is time and experience intensive, it fails to provide information on the viability and source of detected cysts, and extrapolating to get quantitative results is difficult and questionable. These problems are inherent in a detection technique that is dependent upon microscopic examination of specimens.

Work is in progress at HERL and elsewhere on fluorescent antibody (FA) staining techniques (11,12). FA techniques offer the promise of simplifying the detection of cysts in concentrates and they may be useful in differentiating animal and human-source cysts (12). However, currently available FA techniques do not solve the problem of determining viability. Recent reports on the use of the Mongolian gerbil as an animal model (13,14) may allow determining the viability or infectivity of cysts, but the expense and other disadvantages of animal assay systems would remain. Giardia methodology is evolving and the challenge is to develop simpler and better approaches to identifying, detecting, isolating, and enumerating Giardia in water supplies.

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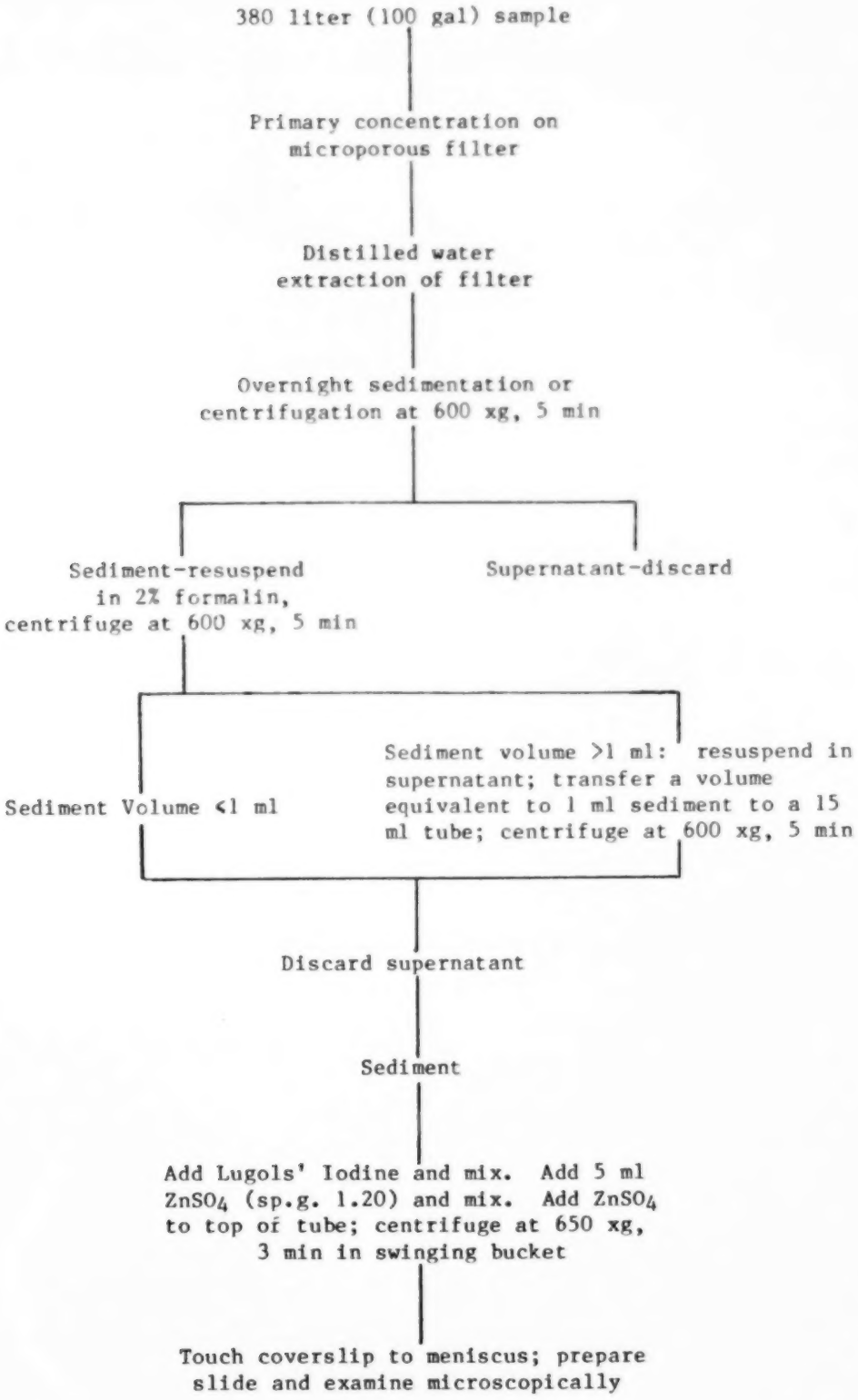
Figure 1. *Giardia* Reference Method Flow Chart


380 liter (100 gal) sample
Primary concentration on microporous filter
Distilled water extraction of filter
Overnight sedimentation or centrifugation at 600 xg, 5 min
Sediment-resuspend in 2% formalin, centrifuge at 600 xg, 5 min
Supernatant-discard
Sediment Volume <1 ml
Sediment volume >1 ml: resuspend in supernatant; transfer a volume equivalent to 1 ml sediment to a 15 ml tube; centrifuge at 600 xg, 5 min
Discard supernatant
Sediment
Add Lugols' Iodine and mix. Add 5 ml $ZnSO_4$ (sp.g. 1.20) and mix. Add $ZnSO_4$ to top of tube; centrifuge at 650 xg, 3 min in swinging bucket
Touch coverslip to meniscus; prepare slide and examine microscopically